Revue suisse Zool.	Tome 82	Fasc. 1	p. 91-100	Genève, mai 1975

On Lithium and Thiocyanate action on embryonic development and metabolism

by

Silvio RANZI

With 5 figures and 2 tables

Dedicated to Professor Hans Mislin.
This work was aided by a research grant of the Consiglio Nazionale delle Ricerche

Summary

Morphological and physiological changes induced by lithium and thiocyanate action are presented and discussed.

Alterations induced in sea urchin and Amphibia embryonic development are well known. Thiocyanate 0.1 M injected in unincubated chick egg, acting up to the stage of primitive streak, induces an enlargement of the notochord.

Lithium inhibits RNA and protein syntheses. Also the breakdown of precursor molecules and the breakdown of synthesized molecules are inhibited.

Some data seem to indicate that NaSCN enhances RNA and protein syntheses.

In a research series we arrived at the conclusion that protein catabolism is at the base of embryonic determination.

Alterations of embryonic development were already well known. For some time the methods to obtain them had also been known.

In the development of sea urchins the alterations are: hyperdevelopment of the rudiments that appear at the animal pole of the egg, at the expense of the vegetal pole (animalization); and hyperdevelopment of the rudiments that appear at the vegetal pole, at the expense of the animal pole rudiments (vegetalization).

In the development of Amphibia: the formation of a large notochord; the reduction of the notochord to total disappearance, whereas the presumptive notochord cells become myotome cells. Also in Amphibia: the development of the nervous system from the presumtive epidermis (the organizer, according to Spemann); and the reduction of the nervous system.

By adding NaSCN to seawater where sea urchin eggs are developing, to the fresh water where Amphibian embryos are developing, or to the culture medium where ex-

planted ectoderm of Amphibia are developing, it is possible to obtain: animalization, large notochord, neural cell differentiation from the ectoderm. *o*-iodosobenzoic acid, $ZnCl^2$ and other substances act in the same way as NaSCN. On the contrary, LiCl, added to the culture medium, induces vegetalization, hypodevelopment of notochord, and inhibits the nervous system.

During our research, the phenomena induced by NaSCN (animalization of sea urchins, enlargement of the notochord of Amphibia, transformation of presumptive epidermic cells into nervous cells in Amphibia) appeared to be related to a protein denaturation phenomenon. We reached this conclusion after a long series of research work, and especially from the following observations:

1. Protein solutions extracted from adult organisms (serum albumin, myosin, actin, etc.) or from eggs or embryos (lipovitellin, lipovitellenin or other, more impure, fractions, such as euglobulin *a*, euglobulin *b*, euglobulin *c*, pseudoglobulin) are denatured by treatment with substances which induce a hyperdevelopment of the above-mentioned rudiments. The viscosity of the solution changes, more reactive groups can be detected; some proteins, such as lipovitellin and lipovitellenin, show, at ultracentrifuge, the formation of subunities; if the protein molecule is fibrillar (myosin) the flow birefringence is lost.

2. The proteins extracted from the embryos, showing malformations induced by NaSCN, are predisposed to a further denaturation induced by urea, or are more easily digested by trypsin and papain.

3. The animalized sea urchin embryos are less resistent to the urea or trypsin action than the control embryos.

4. Urea, the classic protein-denaturing substance, induces animalization, if present in seawater where sea urchin eggs are developing. Urea also induces the development of segments of notochord and nervous rudiments in the ventral-half of young gastrula of Amphibia. In the ventral-half of gastrula, presumptive notochord and nervous system are absent, and these rudiments do not appear in the controls in Holtfreter solution.

Sea urchin vegetalization, reduction of notochord in Amphibia, reduction of nervous rudiments in Amphibia, appeared in our research, induced by a stabilization of the protein molecules; this stabilization preserves the molecules from denaturation. This conclusion arose principally from the following observations:

1. Protein solutions extracted from eggs, embryos, or adult organisms, are inhibited in denaturation if LiCl, or other substances with a similar effect on embryonic development, are added. That is: the viscosity of the protein solutions increases, also for fibrillar proteins, the reactive groups do not increase, at ultracentrifuge subunities do not appear, flow birefringence does not change.

2. Proteins extracted from vegetalized embryos are more resistent to the urea-induced denaturation, and less easily digested by trypsin or papain.

3. Vegetalized sea urchin embryos are more resistent to urea and trypsin than the control embryos.

Therefore, we interpreted the LiCl action as being due to the fact that the stability of a protein molecule is due to an aqueous coat bound to it and the Li ion bound to the molecules is highly hydrating in effect. The aqueous coat protects the protein molecules from denaturation, and from the attack of the proteolytic enzymes. This coat preserves the molecules from metabolization, with a process which, at the molecular level, is the

process that the old experimental embryologists called developmental inhibition, on the basis of their microscope observations.

If we refer the above to normal development, we can advance a tentative interpretation. Pre-existing protein molecules are metabolized to obtain the protein synthesis related to the formation of the sea urchin ectoderm rudiments of the animal pole, as well as to the formation of Amphibian notochord and neural tube. This protein breakdown is preceded by a denaturation process. If we add, for example, NaSCN or *o*-iodosobenzoic acid, this denaturation process is enhanced. The rudiments in question are larger. If the denaturation is inhibited by a Li aqueous coat, the development of the rudiments is reduced, and they eventually disappear (loss of ciliar tuft in vegetalized sea urchins, transformation of notochord cells into somite cells in Amphibia). All these data are reported by Ranzi (1962).

MORPHOLOGICAL OBSERVATIONS

Subsequent research was carried out to see if the LiCl action can be identified with the action of an inhibitor of the protein synthesis. A system as complicated as that of chick embryo at the primitive streak stage was investigated.

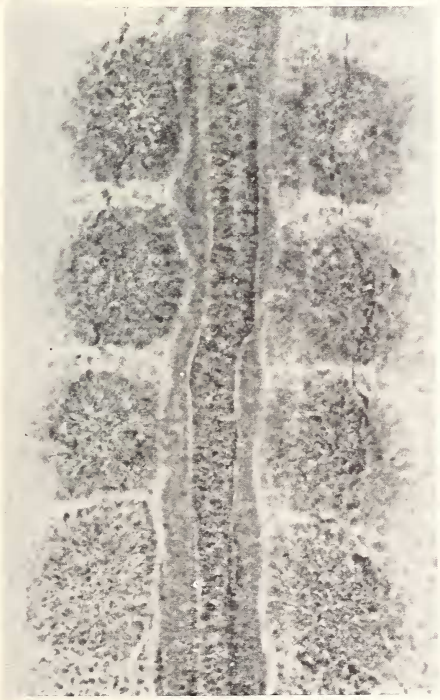

Fig. 1.

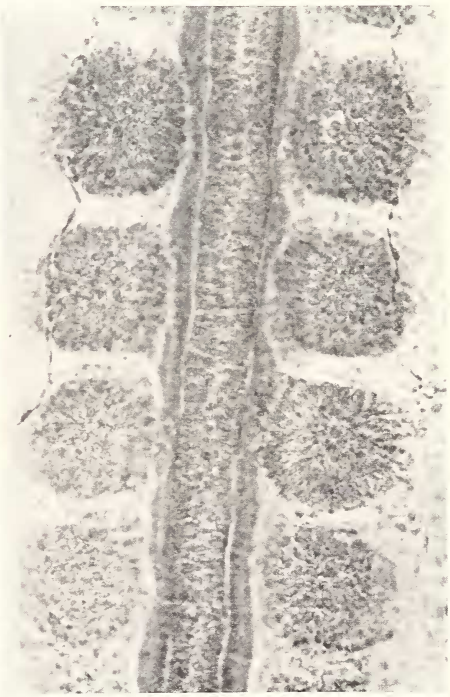

Fig. 2.

Fig. 1. — Chick embryo of 11 somites: notochord and somites (× 200)

Fig. 2. — Chick embryo of 11 somites treated in egg with NaSCN. The notochord is larger than in the control of Fig. 1 (× 200)

Morphological alterations induced by LiCl in chick embryo development (somite fusion on the median line with notochord reduction, omphalocephalic, cyclopic and platyneuric embryos) are completely different from the malformations induced by transcription and translation inhibitors. Actinomycin D and daunomycin, inhibitors of the synthesis of DNA-dependent RNA, induce malformations in embryos at the primitive streak stage. These malformations are: reduced neural tube with more evident reduction

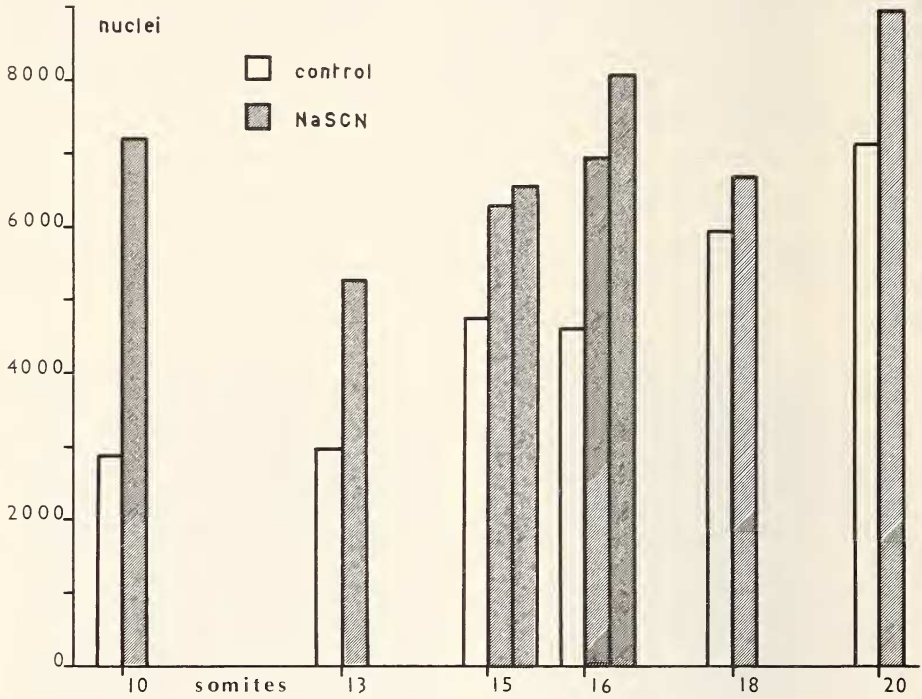

FIG. 3.

Number of nuclei in notochord of controls and of NaSCN-treated embryos.

of the fore-brain, the nervous system reduction can arrive at total disappearance, but no cases of platyneuric embryos have been observed. Somites are inhibited, notochord generally present and well developed, heart well developed, sometimes embryos have been observed with just the heart. Puromycin, which inhibits the translation of information from RNA to proteins, induces, in the embryo at the stage of primitive streak, a general inhibition of all organs, including the heart (LEANI COLLINI and RANZI, 1967). The conclusion that the Li action is different from the action of the actinomycin D is confirmed by the research work of RUNNSTRÖM and MARKMAN (1966) on sea urchin embryos. These authors observed that actinomycin D inhibits the entoderm differentiation in exogastrulae. Consequently, it is impossible to conclude that actinomycin D vegetalizes the sea urchin embryo (see LALLIER, 1963).

Acting at the very early stage, before notochord determination with NaSCN, it is possible to obtain a percentage (17%) of embryos with larger notochords than the

controls [1] (figs. 1, 2, 3). In more advanced stages, it is possible to see a somite degeneration (fig. 4). The phenomenon of transformation of mesoderm cells into notochord cells that is described in Amphibia is likely to happen in chick embryo; it is possible, however, to see an enlargement of the notochord, and a somite degeneration.

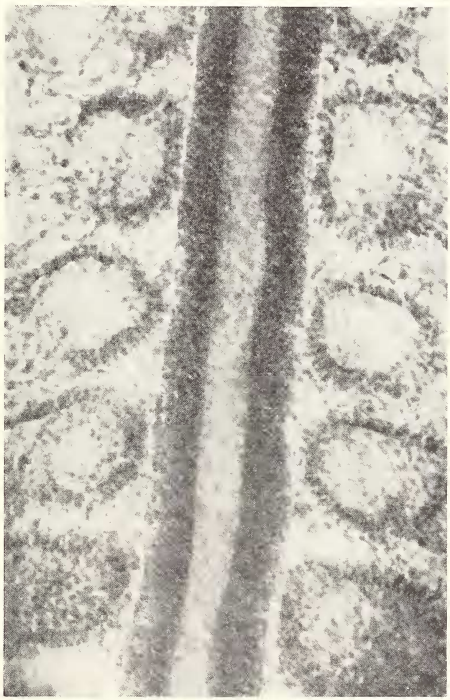

FIG. 4.

Chick embryo of 17 somites treated in egg with NaSCN.
The somites are degenerating (× 200)

EMBRYONIC METABOLISM

In *Xenopus* embryos treated with 0.01 M LiCl, starting from the stage of young blastopore, the RNA synthesis is less active than in the controls. There is less RNA in treated embryos than in the controls. This reduced synthesis is accompanied by a diminished breakdown. The diminution of breakdown is more easily recognizable in embryos developed at lower LiCl concentrations, concentrations not strong enough to induce morphological malformations, because in these embryos the synthesis is less

[1] In fertilized eggs 1 ml of albumen is removed, and substituted by 1 ml of 0.1 M NaSCN solution in distilled water. In the controls, 1 ml of albumen is substituted by physiological solution. After these operations, the eggs are incubated. One day after, at the primitive streak stage (stage 4, according to HAMBURGER and HAMILTON), or at the stage of head-process (stage 5), they are explanted and put in *in vitro* culture, according to the technique of NEW (1966). The nutritive medium used is fluid albumin and Pannett-Compton solution, without NaSCN.

reduced. If these embryos are developed in the presence of marked precursors, the amount of C14 incorporated in RNA is higher than in the controls. The sedimentation profiles show marked fractions, 4, 18 and 28 S; more active than in the controls. A 22 S fraction, impossible to recognize in these latter, appears, it is probably a precursor of 18 S fraction (Leonardi Cigada et al., 1972).

In chick embryos treated with LiCl, using the methods followed by Leani Collini and Ranzi (1967), the incorporation of tritiated uridine was studied, with the autoradiographic method (table 1). It is thus possible to see the RNA synthesis. In notochord and somites of Li-treated embryos, the incorporation was lower than in the controls. The incorporation in the nervous system rudiments was greater if referred to 100 μ^2, than in the controls, but the nervous rudiment of these embryos was smaller than in the controls, and so were the cells. Consequently, it is possible to arrive at the same conclusion as Flickinger et al. (1969), regarding Amphibian embryos: that cells treated with LiCl synthesize less RNA than the control cells. The same was observed by Volm et al. (1970) for *Tetrahymena* culture treated with 0.01 M LiCl. Vegetalized sea urchin larvae synthesize less RNA than the control larvae (Berg, 1968).

Table 1.

Incorporation of tritiated uridine (traces per 100 μ^2) 25 μc of tritiated uridine per embryo at 38.5° C in 1 ml of physiological solution. The chick embryos remained in tritiated uridine 1 hour in the LiCl experiment, 24 hours in the NaSCN experiment.

	Control	LiCl	Control	NaSCN
Neural plate	46.3 ± 3.63	58.8 ± 3.50	72.2 ± 1.74	58.2 ± 1.13
Notochord	41.9 ± 3.40	30.6 ± 1.72	67.6 ± 1.68	55,7 ± 1.31
Somites	46.1 ± 3.93	40.8 ± 1.42	71.3 ± 2.01	56.2 ± 1.56

Experiments of Leonardi Cigada et al. (1973) on *Xenopus* embryos show an increase of CO^2 incorporation in RNA, induced by NaSCN. In autoradiography (table 1) chick embryos treated with NaSCN show that the incorporation of tritiated uridine does not increase. Bäckström (1959) showed in animalized sea urchin larvae values slightly higher than in the normal larvae. Flickinger et al. (1970) and Volm et al. (1970) were able to observe an increase of RNA synthesis induced by $NaHCO^4$ or NaSCN in Amphibia or *Tetrahymena* cells.

If we consider the proteic metabolism in the first stages of development, we can recognize three different phenomena: the digestion of the reserve material, the synthesis of new proteins, and the breakdown of the latter. These three phenomena have been analysed during the development of Li-treated *Xenopus* embryos.

1. Eggs labelled with tritiated leucine during the growth stage in the female, developed into embryos that showed more tritium if treated with 0.01 LiCl at the beginning of gastrulation (Ranzi and Vailati, 1971). That is, the breakdown of reserve material is lower after LiCl treatment.

2. Embryos at the end of neurulation, i.e., when protein synthesis is very active, are treated with daunomycin 5 mg, puromycin 2.5 mg, or LiCl 0.01 M, and labelled

with C14 protein hydrolysate. The daunomycin, transcription poison, does not change sensibly the sedimentation profile in its optical density and radioactivity. LiCl, instead, and puromycin, translation poison, induce considerable inhibition of C14 fixation on ribosomes (De Bernardi et al., 1969). That is, LiCl inhibits the protein synthesis. This

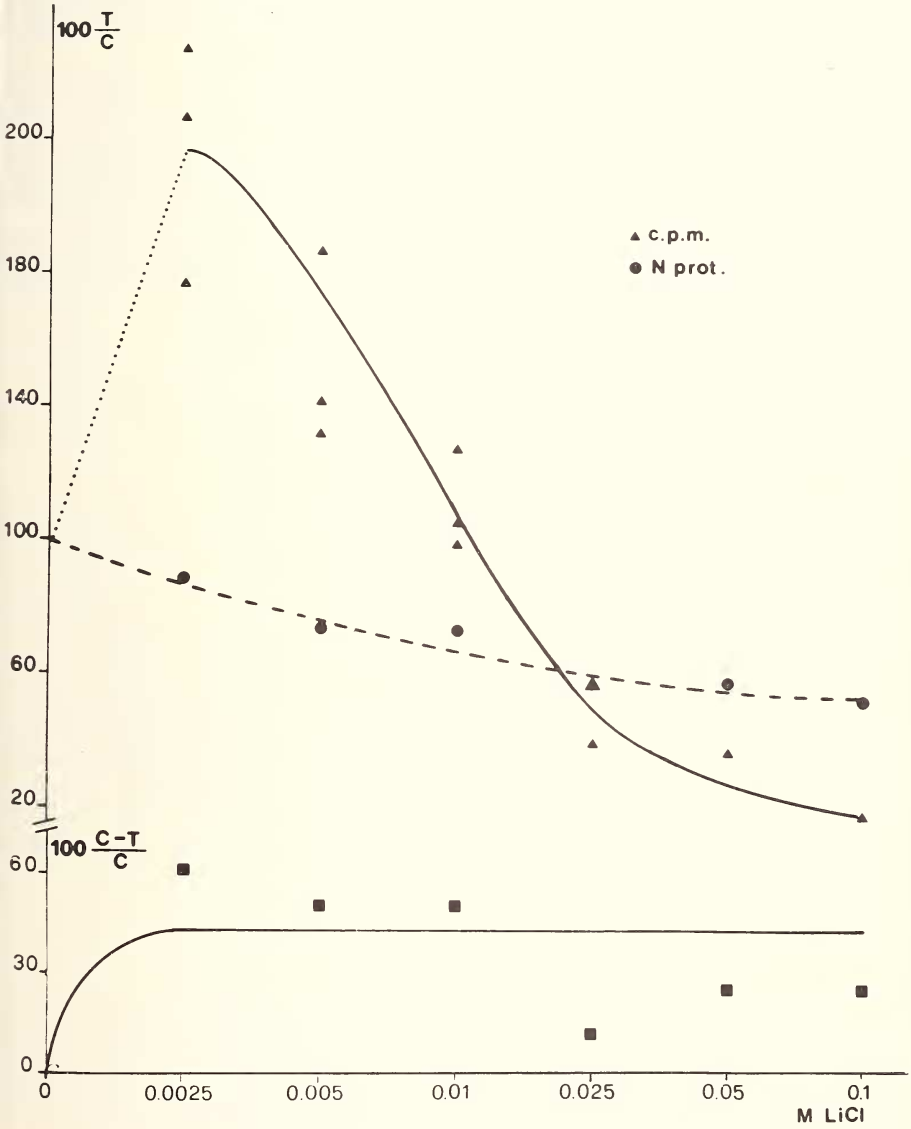

Fig. 5.

Spectrophotometric readings (circles, broken line) and C14 incorporation (triangles, unbroken line) in protein extracted from *Xenopus* embryos treated with different LiCl concentrations. Control equal to 100. Below (squares, unbroken line): inhibition of the trypsin digestion of LiCl-treated proteins.

conclusion is corroborated by the fact that embryos treated with LiCl have less protein than the controls (Leonardi Cigada et al. 1973).

3. Embryos are put in 0.0025 M LiCl at the beginning of gastrulation, i.e., they are put in a solution that is not active in inducing malformations. They are then put in a solution of C14 labelled protein hydrolysate. It is possible to see that in these embryos there is more labelled material than in the controls, that is, the breakdown of synthesized proteins is less than in the controls.

This analysis of the protein metabolism of the embryo leads to the conclusion that LiCl at lower concentrations, i.e., in concentrations inactive at a morphological level, inhibits the protein breakdown (yolk utilization and breakdown of newly-built proteins) more than the synthetic processes, also inhibited. The inhibition of these processes is, on the other hand, greater at concentrations active in determining morphological alterations (fig. 5).

Also in autoradiographic research work on chick embryos treated with LiCl, it is possible to see that the incorporation of tritiated leucine is inhibited by LiCl (table 2).

Table 2.

The same experiments as in Table 1, using tritiated leucine

	Control	LiCl	Control	NaSCN
Neural plate	72.2	58.2	76.7	74.9
Notochord	67.6	55.7	70.7	71.9
Somites	71.3	56.2	74.4	74.0

Some data are available on NaSCN action on protein metabolism. Leonardi Cigada et al. (1973) were able to observe in *Xenopus* embryo an increase of protein hydrolisate incorporation. Lanzani Maci et al. (1972) do not observe a variation of the incorporation of tritiated leucine in chick embryo culture in vitro (table 2). Volm et al. in *Tetrahymena* cells observed an increase of incorporation of tritiated leucine induced by 0.01 M NaSCN. Bäckström (1959) found a larger quantity of glucose-6-phosphate dehydrogenase in sea urchin animalized larvae (the animalizing agent used was *o*-iodosobenzoic acid).

DISCUSSION

The studies reported here show that the morphogenetic actions induced by lithium, and the malformations induced by substances that denature the protein, such as NaSCN, can be put in relation to the transformations induced in the cellular proteins. These proteins are denatured on the appearance of ectoderm in sea urchins, or notochord in Vertebrates, or when the nervous system is induced in Vertebrates. Instead, the proteins appear stabilized when entoderm develops in sea urchins, notochord or nervous rudiments are reduced in Vertebrates.

Li-treated embryos show reduced transcription and translation processes; but the poisons of transcription and translation are unable to induce in chick embryos the same alterations as LiCl.

The syntheses of RNA and proteins, and in general the whole metabolism of these substances, are inhibited by LiCl action.

In experiments of LEONARDI CIGADA et al. on *Xenopus* embryos and of VOLM et al. on *Tetrahymena*: RNA and protein syntheses increase. LANZANI MACI et al. do not see this increase in autoradiography of chick embryo. Other research work of FLICKINGER et al. and of BÄCKSTRÖM agrees with this increase.

RÉSUMÉ

Des modifications morphologiques et physiologiques dues à l'action du lithium ou de NaSCN sont illustrées et discutées.

Des altérations provoquées au cours du développement des oursins ou des batraciens sont bien connues. NaSCN inoculé à raison de 10 ml. 0,1 M dans l'œuf de poulet pas encore incubé et agissant jusqu'au stade de la ligne primitive provoque sur un certain pourcentage d'embryons (17%) un grossissement de la corde aux dépens des somites.

Le LiCl empêche la synthèse d'ARN et de protéines. La destruction des molécules préexistantes et celle des molécules néoformées est aussi interrompue.

Dans de nombreux cas la synthèse d'ARN et de protéines augmente sous l'action de NaSCN.

ZUSAMMENFASSUNG

Morphologische und physiologische Veränderungen, verursacht durch Einwirkung von Lithium oder Rhodanid, werden dargestellt und diskutiert.

Gut bekannt sind Veränderungen in der Entwicklung von Seeigeln und Amphibien. Injektionen von 1 ccm 0,1 M NaSCN in nicht bebrütete Hühnereier — die Wirkung der Rhodanid dauert bis zum Stadium des Primitivstreifens an — rufen bei einem gewissen Prozentsatz der Embryonen (17%) eine Vergrösserung der Chorda auf Kosten der Somiten hervor.

Lithium hemmt die RNA-und Protein-Synthese; ebenfalls gehemmt wird die Zerlegung der vorher bestehenden Moleküle und der neu gebildeten Moleküle.

Die RNA- und Protein-Synthese wird in vielen Fällen durch die Einwirkung von Rhodanid erhöht.

REFERENCES

BÄCKSTRÖM, S. 1959. Activity of glucose-6-phosphate dehydrogenase in sea urchin embryos in different developmental trends. *Expl. Cell Res.* 18: 347.

BÄCKSTRÖM, S. 1959. Changes in ribonucleic acid content during early sea urchin development. *Ark. Zool.* (2) 12: 339.

BERG, W. E. 1968. Effect of Lithium on the rate of protein synthesis in the sea urchin embryo. *Expl. Cell Res.* 50: 133.

DE BERNARDI, F., M. LEONARDI CIGADA, R. MACI and S. RANZI. 1969. On protein synthesis during the development of Lithium treated embryos. *Experientia* 25: 211.

FLICKINGER, R. A., M. R. LAUTH and P. J. STAMBROOK. 1970. An inverse relation between the rate of cell division and RNA synthesis per cell in developing frog embryos. *J. Embryol. exp. Morph.* 23: 571.

HAMBURGER, W. and H. L. HAMILTON. 1951. A series of normal stages in the development of the chick embryo. *J. Morph.* 88: 49.

Lallier, R. 1963. Effet de l'actinomycine D sur le développement normal et sur les modifications expérimentales de la morphogenèse de l'œuf de l'oursin *Paracentrotus lividus*. *Experientia* 29: 572.

Lanzani Maci, R. e C. Sotgia. 1972. Cambiamenti della precoce determinazione embrionale e sintesi di acido ribonucleico e di proteine nell'embrione di pollo. *Atti Acc. naz. Lincei Rc.* (8) 53: 602.

Leani Collini, R. e S. Ranzi. 1967. Effetto di actinomicina D. daunomicina, puromicina e litio cloruro sui primi stadi dello sviluppo embrionale del pollo. *Atti. Accad. naz. Lincei. Memorie.* (8) 8 (3): 34.

Leonardi Cigada, M., F. Laria De Bernardi e A. M. Bolzern. 1972. Sintesi di RNA in embrioni di *Xenopus* trattati con LiCl. *Atti. Accad. naz. Lincei Rc.* (8) 52: 93.

Leonardi Cigada, M., F. Laria de Bernardi e M. Scarpetti Bolzern. 1973. Sintesi di proteine in embrioni di Xenopus laevis trattati con LiCl. *Istituto Lombardo (Rend. Sc.)* B, 107: 117.

Leonardi Cigada, M., F. Laria de Bernardi e M. Scarpetti Bolzern. 1973. Sintesi di acido ribonucleico e di proteine in embrioni di Anfibi trattati con solfocianuro. *Atti Acc. naz. Lincei Rc.* (8) 55: 755.

New, D. A. T. 1966. The culture of Vertebrate embryos. *Acad. Press, N.Y.*

Ranzi, S. 1962. The protein in embryonic and larval development. *Adv. Morphog.* 2: 211.

Ranzi, S. 1965. Problèmes d'immunochimie et de la différentiation protéique dans le développement des Oursins, des Batraciens et d'autres animaux. In: Wolff. *New Methods in Embryology. Hermann, Paris* p. 47.

Ranzi, S. e G. Vailati. 1971. Azione del cloruro di litio sul metabolismo proteico dell'embrione di *Xenopus*. *Atti. Accad. naz. Lincei Rc.* (8) 50: 473.

Runnström, J. and B. Markman. 1966. Gene dependency of vegetalization in sea urchin embryos treated with Lithium. *Biol. Bull.* 130: 402.

Vailati, G., P. Vitali e S. Ranzi. 1972. Azione di solfocianuro di sodio nei primi stadi dello sviluppo dell'embrione di pollo. *Atti Acc. naz. Lincei Rc.* (8) 53: 594.

Volm, M., K. Wayss und V. Schwartz. 1970. Wirkung von Lithium und Rhodanidionen auf die Nukleinsäure und Protein Synthese bei *Tetrahymena pyriformis* GL. *Wilhelm Roux Arch. Entw. Mech. Org.* 165: 125.

Author's address:

Laboratorio di Zoologia
Via Celoria 10
I-20133 Milano
Italie